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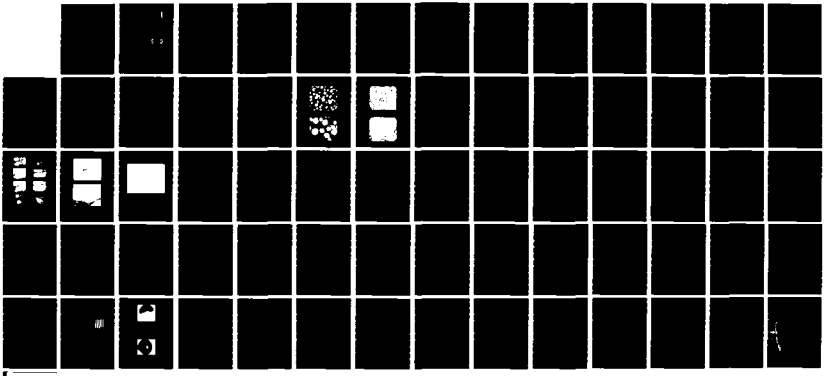

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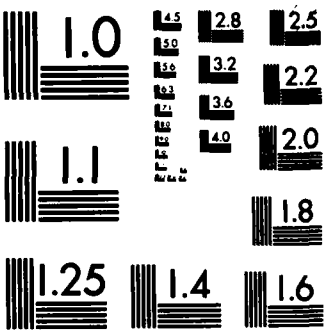

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# LASER INDUCED DAMAGE IN THE EYE

Final Report

William J. McGowan

May 1977

Supported by

US Army Medical Research and Development Command
Fort Detrick, Frederick, Maryland 21701

Contract No. DAMD17-76-G-9419

University of Western Ontario
London 72, Canada

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THE UNIVERSITY OF WESTERN ONTARIO

# LASER INDUCED DAMAGE IN THE EYE:
# STUDY OF ENERGY DEPOSITION IN THE RETINA

FINAL REPORT FOR THE GRANT PERIOD

June 1, 1976 to May 31, 1977


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May, 1977.

## INTRODUCTION

With this report we summarize the developments and accomplishments which occurred during year two of grant No. DAMD17-76-G-9419, Laser Induced Damage in the Eye. As proposed, the project was to focus upon a detailed study of ultra-structural damage to the photo receptors and a complementary study of the optical properties of these receptors. As a result we have studied retinal damage and repair at both supra-threshold and low-level exposure conditions. Significant progress is associated with

1) the near completion of our suprathreshold studies
2) the further development and use of phase grating laser exposure techniques
3) the extensive use of high resolution histological examination techniques for the evaluation of ultrastructural alterations of the receptors at low coherent light levels
4) the completion of optical modelling studies and
5) the development of X-ray lithography which should permit time resolved spectroscopic as well as microscopic studies of live retinal cells to be undertaken next year.

In figure 1, a chart, we outline more completely the major activities of the research team this year. The developments in each area are discussed in greater detail in the following sections. Most of the work completed during the year is given in detail as papers either published or prepared for publication, many of which are appended to this report. The appendix also includes further details on aspects that will form the basis of

## STUDIES OF LASER INDUCED DAMAGE IN THE EYE

### PROJECTS NEARLY COMPLETED

1. Characterization of Suprathreshold Damage
2. Epiretinal Membrane Formation
3. Theoretical modelling of photoreceptor optical properties

### STUDIES CONTINUING

4. Ultrastructural alterations at low light levels
5. Baseline studies of normal primate retina
6. Monochromatic Light effects on Retina
7. X-ray lithography - microscopy/spectroscopy of live cells

### OTHER ACTIVITIES

8. Papers and colloquia delivered
9. Papers published
10. Workshop group
11. Working group "Chronic Light Damage to the Eye"
12. Visits of David H. Sliney and John Marshall

Fig. 1. Outline of the major activities carried out by the U.W.O. research team.

future publication resulting from the present studies.

## 1. CHARACTERIZATION OF SUPRATHRESHOLD DAMAGE

A major study on the characterization of laser-induced damage in the rabbit retina at suprathreshold irradiance levels using scanning electron microscopic observation has been completed during the past year. The SEM observations of normal and lased retina and pigment epithelium (summarized in two papers appearing in the Canadian Journal of Ophthalmology and in Investigative Ophthalmology appended to the 1975-76 report)have been used to good advantage. We also have significant confirmation that pigment epithelial cells can regenerate following laser irradiation damage.

## 2. INVESTIGATION OF THE FORMATION OF EPIRETINAL MEMBRANES

As an extension of the study of suprathreshold damage mechanisms, we explored the possibility of inducing and characterizing epiretinal membranes in rabbit eyes following laser exposure. This investigation was spurred by our early observation of the formation of membrane-like structures in the rabbit eye at high-exposure levels. The formation of such membranes is important in the clinical applications of laser irradiations of the human eye since the formation of epiretinal membranes can lead to retinal detachment originating at the site of membrane formation. This can lead to catastrophic failure of the clinical treatments.

We used suprathreshold laser lesions sufficient to

penetrate the full thickness of the retina, and examined these retinas by scanning electron microscopy. The membranes we saw in our early work we now consider to be the rolled-up membrane of the vitreal-retinal interface and the cobweblike strands have been shown, by experiment, to be vitreal condensations. Dr. M.J. Hollenberg, University of Calgary, who processed some of these tissues, concurs in this view. The techniques learned in this work have proved invaluable and will continue to be applied to the characterization by SEM of laser-induced alterations.

## 3. THEORETICAL BACKGROUND OF OPTICAL PROPERTIES OF THE RECEPTORS AS THEY AFFECT FUNCTION AND OPTICALLY INDUCED DAMAGE

Despite the tremendous strides of recent years in electro-physiology and microspectrophotometry we are as yet still a long way from understanding the basic transduction process whereby incident light is converted into electrical signal in the photoreceptors. Nor is it understood how light, at sufficiently high intensities, might be directly toxic to the photoreceptors, the result suggested by the observations of Adams, Beatrice, and Bedell (1972) of ultrastructural alterations, produced by retinal exposure to relatively low coherent laser light levels. Whatever the interaction processes in the receptors leading to transduction and to ultrastructural alterations might be, it is clear that they will depend directly on the intensity of the radiation field at the receptors.

A major factor influencing the interaction processes of light in the receptors is, of course, the nature of the light absorbing substance contained in the receptor outer segments. The characteristic absorption spectra and density of the receptor photopigments determine what portion of the radiation energy actually present in the receptors is absorbed and subsequently produces a physiological effect and (potentially at least) ultrastructural alterations. In the traditional view of visual science where the photoreceptors are pictured simply as containers for the photopigments - containers whose details of dimensions and construction are essentially ignored - light is pictured as illuminating the receptor interior uniformly irrespective of the optical wavelength.

However, basic optical physics dictates that this picture is not adequate. The geometrical optics description of light propagating along a fibre by total internal reflection at the interface between the fiber and surrounding medium is valid only when that fibre is large in cross-section compared to the wavelength of the light being transmitted. This is not the case for visible light propagating along the outer segments of the primate cones. The diameter of foveal cones, for example, is only the order of two times the wavelength of visible light. For fibres this small one must turn to the wave optical description in order to characterize the transmission properties determining how light of different wavelengths can illuminate the interior of such a fibre. The details of this physical description are rather complicated, and were summarized in

section 4 of the 1975-1976 annual report. This material is to be published as a series of articles.

In terms of the present studies of laser-induced ultrastructural alterations, the existence of strong wavelength dispersive effects in the retinal cones can be expected to influence the kinds of low-level damage produced in the cones by light of different wavelengths. The ultrastructural alterations can be expected to depend on both the optical transmission characteristics of the receptors which determine how the incident optical power is distributed within the receptors as well as the nature of the receptor photopigments determining what portion of the energy present is actually absorbed.

Thus, depending on the absorption spectra and physical distribution of cone photopigments we might expect to see ultrastructural alterations following exposure to long-wavelength light to occur primarily at the wide proximal portion of the cones and the damage from short wavelength light to be distributed over the entire length of the cones (or possibly confined to the smaller diameter regions of the cones if different photopigments are differentially segregated within the cone outer segment,for example). During the past year the developments on these theoretical studies have included the writing of a computer program for calculating the energy deposited along the length of an absorbing cone as a function of the optical parameters (its dimensions, refractive indices, and absorption coefficients of any photopigments). In addition a number of relationships between the receptor optical properties and various aspects of

color vision have been worked out. These include:

a. Design for a cone spectrometer device -

The development of a model for the construction of a physical analogue of the cone spectrometer principle. The basic aspects of information coding in this model is similar to some of the major observational characteristics in human color vision including trichromacy (and dichromacy in a reduced version) and the existence of a temporal color code producing the same color perceptions as in human vision for subjective color phenomena such as the well-known (but as yet not understood) Benham's Top.

b. Closure of the cone spectrometer "color circle" -

Because sufficiently short wavelengths will excite higher order modes in an optical fiber and since these will then propagate along a tapered fiber much like longer wavelengths in the fundamental mode, a cone spectrometer's operating range will be closed. The illumination pattern of a cone with violet light incident on it will thus be very similar to that of a mixture of blue light (light propagating to the lowest order mode) plus a small "red" component (light propagating in a higher order mode). That we may identify this mechanism as playing a role in the characteristic appearance of violet light in human vision is suggested by quantitative comparison.

Second order transmission in an optical fiber occurs for values of the waveguide parameter V of 2.405 or larger. This waveguide parameter is given by

$$V = \frac{\pi d}{\lambda} (n_1^2 - n_2^2)^{\frac{1}{2}}$$

where d is the fiber diameter, $\lambda$ the free-space wavelength of the optical radiation, and $n_1$ and $n_2$ the refractive indices of the fiber and its surround, respectively.

The best available estimates and measurements for the refractive index difference (essentially the fiber's numerical aperture) for primate cones is $(n_1^2 - n_2^2)^{\frac{1}{2}} = 0.30$ (Barer, 1957; Sidman, 1957; Liebman, 1972). Thus for human cones, $V \simeq d/\lambda$. For human foveal cones with a maximum outer segment diameter of between 1.0 to 1.1 microns, second order mode transmission ($V = 2.405$) will occur for optical wavelengths between 416 and 460 nm, corresponding to the region where the violet perception (as blue light plus a red component) is observed for human vision.

c. The development of the relationship between the various kinds of eye micromovements (tremor and saccades) and an encoding scheme for color information as time information in the model of a. above, and relating of these to major forms of color defective vision as well as the phenomena of foveal "blue blindness".

The above developments are to be submitted as a series of papers on both the physical principle of cone spectrometer operation and a relationship between cone receptor shape and cone function.

## 4. STUDY OF ULTRASTRUCTURAL ALTERATIONS AT LOW COHERENT LIGHT LEVELS IN THE PRIMATE RETINA

The preliminary phases of this major aspect of the experiment have been completed during the past year. These have included:

a. the development of positioning equipment for the holding of primates and the positioning of their retinas for laser irradiations.

b. tissue handling and preparation techniques for the microscopic examination of the primate retina.

c. demonstration of the optical transform technique for retinal irradiation in the primate retina.

d. The irradiation of three monkeys for the laser-damage studies have thus far been completed.

A technical note on the optical transform technique for laser irradiation has been prepared for publication and is appended to the report. In addition to its potential for increasing the amount of data available from each test eye, the optical transform technique makes possible the precise positioning of laser irradiations too small to be detected by usual observation methods. The technique may also have some important clinical applications.

## 5. BASELINE STUDIES OF NORMAL PRIMATE RETINA

To provide a baseline for studies of ultrastructural alterations induced by laser light damage, we have undertaken a study of normal primate retina by transmission electron microscopy utilizing transverse sections of the retina. The basic aim of this study has been the determination of the physical dimensions and form of the retinal receptors. These features determine the optical properties of the receptors which limits how radiant energy can illuminate (and potentially damage) the receptors. Data on the appearance and dimensional parameters of the photoreceptors have a bearing on the theoretical interpretation of ultrastructural alterations as a function of irradiating wavelength, as well as providing a standard by which "alterations" of normal structure can be measured.

This method of histological study (employing transverse sections through the receptor layer) is yielding a wealth of important new information on receptor structure and organization. These observations include:

a. The confirmation of the existence of a slight tapering of the foveal cones of the primate retina. The existence of a foveal cone taper is of important theoretical significance in connection with optical models of retinal cone function and of laser-induced damage studies. The data available in the literature on the shape and dimensions of the foveal cones is both scanty and rather controversial. A paper on the foveal cone shape and its theoretical significance is planned for the next grant period.

b. Details on the structure and organization of the retinal receptors of the primate retina including:

i. Information on the relative distribution of rods and cones across the retina

ii. The relative sizes of receptors and interreceptor spacings throughout the retina.

iii. The dimensions, shapes, number and positioning of the calycal processes and their relationship to the inner and outer segments of the receptors (Figs. 2-3).

iv. The shape of the inner segment and its marked surface ridging. (Fig. 3-4.

v. The ciliary connective and "the ciliary backbone" alongside the outer segment. (Fig. 2)

vi. Observations on the cone "fins" of the inner segments near the level of the outer limiting membrane (Fig. 5).

vii. Information on the receptor association with the villous processes of the retinal pigment epithelium including a cone "spur" or sleeve-like extension (devoid of discs, from the distal cone tips to the pigment epithelium).

These observations are significant developments towards the understanding of the relationship between receptor structure and function. A review paper on cone structure will emerge from these studies. Part of our planned studies of receptor cell processes will include an examination of the calycal processes with enough resolution to determine whether or not there are actin strands within the calycal processes. The presence of actin would be a strong presumptive indication of the existence

FIGURE LEGENDS

Fig. 2. Transverse section through the photoreceptors in a parafoveal area of Maccaca fascicularis retina showing cones and rods. The cones (c) are of larger diameter than the rods (r) and they are surrounded by a prominent array of calycal processes (ccp). In this photomicrograph they number 19-22. In others the numbers seen varied from 13 to 25. It has not yet been established whether the numbers diminish with distance sclerally from the inner segment junction; or whether the numbers change with retinal zones or are haphazard. The ciliary backbone (cb) is very prominent in cones, and the cilium is usually found more scleral in position in the rods (rci) than in the cones (cci).

Note the marked ridging of the surface of the inner segment (*), more marked in the cones than in the rods (r).

In this micrograph, one can see mainly rod inner segments (ris) and cone outer segments (cos) indicating that the rod inner segments project further towards the pigment epithelium than do the cone inner segments. There are a few cone outer segments of reduced diameter present (1,2,3,4) with small calycal processes, few in number, which would indicate that the cones here taper, and so do their calycal processes, which are eventually lost (4).

X 5,700

Fig. 3. Transverse section of the retina with cone inner segments (cis) showing the marked surface ridging found in the area of the cilium (ci). There are some calycal processes present (arrow) near the cilium. Calycal processes surround the outer segment only as far as the ciliary backbone (arrow heads). Some rod inner segments (ris) are present with irregular shapes, and also rod outer segment (ros) with lobulated outline and a few small calycal processes.

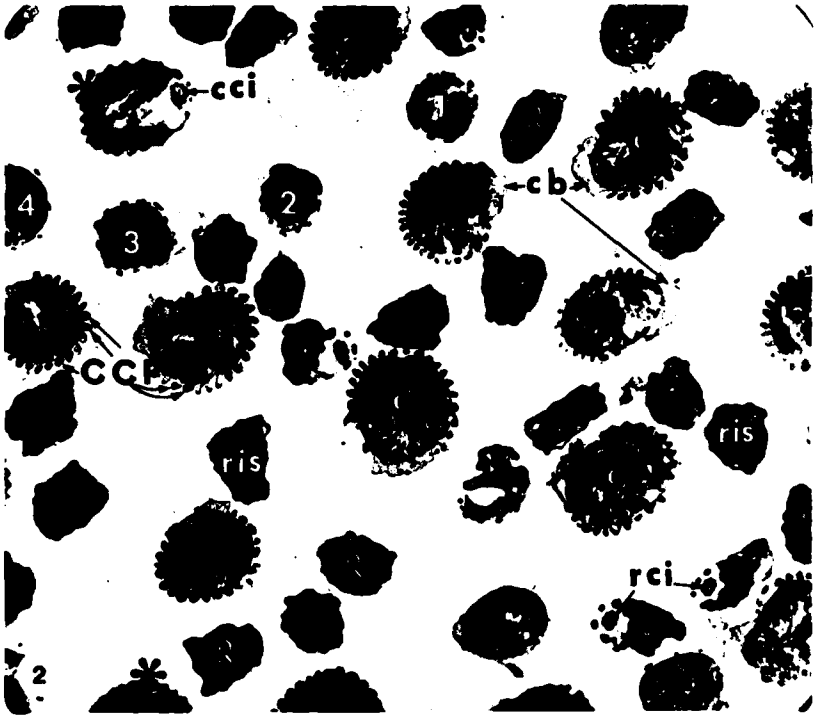
X 9,200

Fig. 4. A transverse section through the rods and cones of the Maccaca fascicularis retina. This is very near the center of the fovea - very few rods are present. A pattern can be constructed of one central cone surrounded by six or seven cones; or they can be viewed as arranged in short rows on a grid pattern. The outlines of both the rods and cones are irregularly ridged. Note the differences in diameter of rods (r) and cones (c) at this level.

X 2,300

Fig. 5. A transverse section through the inner segments of the foveal cones [only 2 rods (r) are present] just scleral to the outer limiting membrane. The cones (c) bear numerous fine lateral projections ("fins") which interdigitate with those of their neighbours to appear gear-like. Note the multiple units of the Golgi apparatus (G).

X 5,700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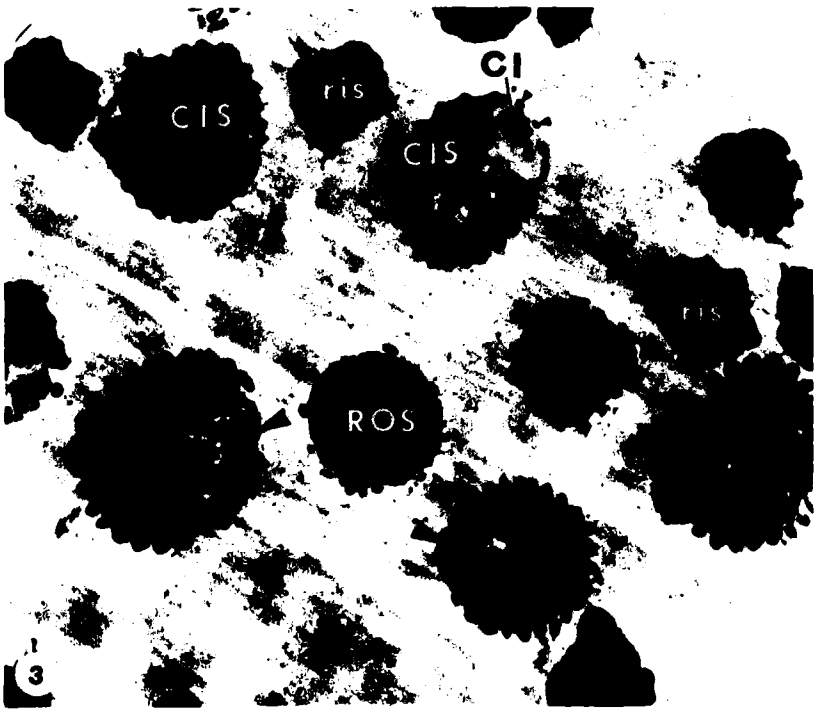
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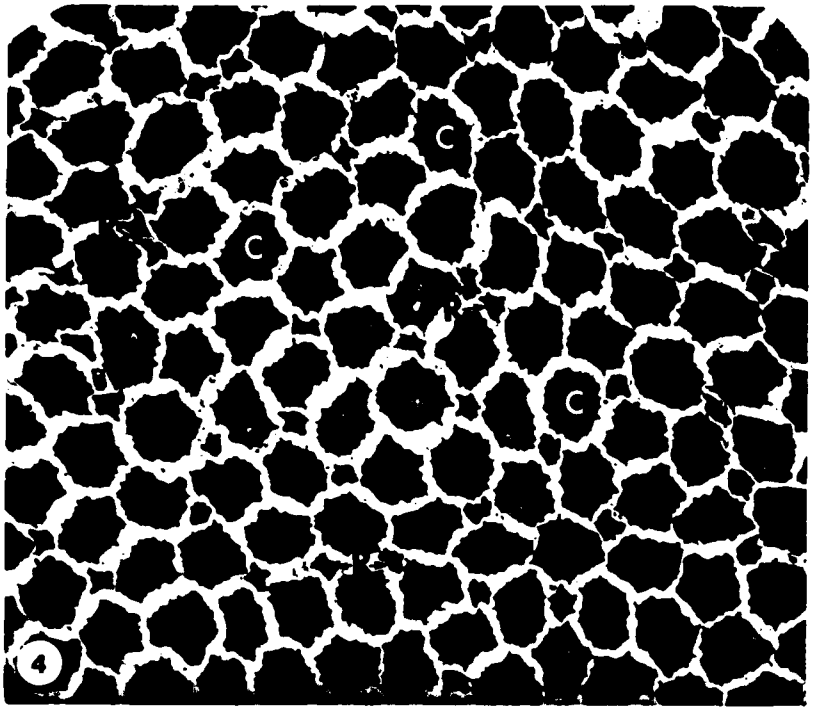
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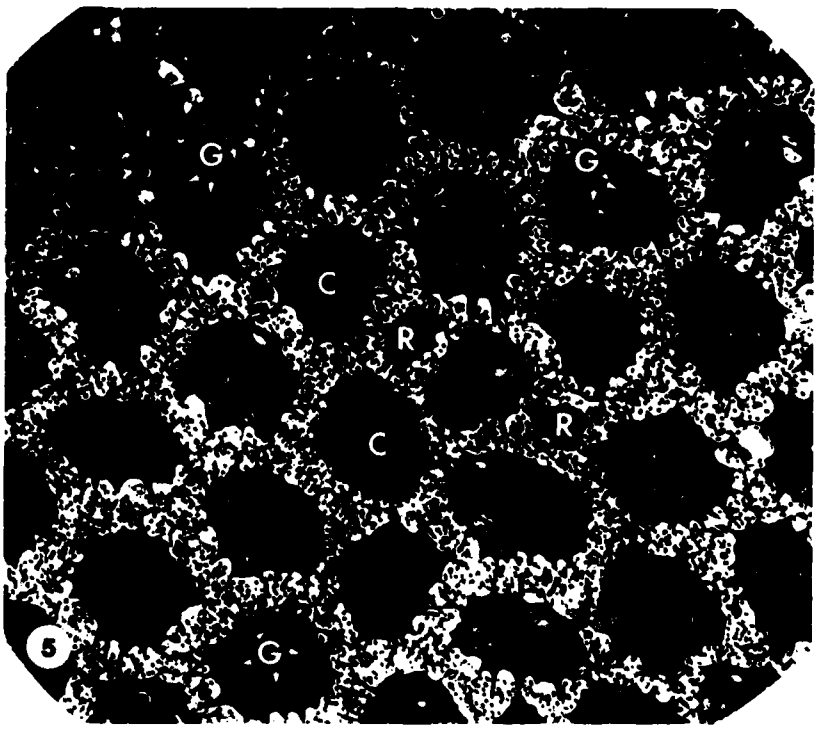
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of mechanical movement of the processes. Such an observation would be highly significant. A paper on the accessory processes of the retinal cones of the primate retina is also planned for the next contract period.

c. The observation and characterization of abnormal features in so-called "normal" areas of the retina of human eyes enucleated for choroidal melanoma. Such observations are important in assessing the suitability of such eyes for studies of ultrastructural alterations since the observed departures from normal appearance of the retina would interfere with detection of alterations at low exposure levels.

Laser lesions have been placed in the subject eyes and await electron microscopic observation (they have already been prepared for such observations) for their evaluation. The altered appearance of test eyes at the ultrastructural level can be expected to influence in only a minor way the damage process operating at higher exposure levels. A paper on the abnormal appearance of such eyes at the ultrastructural level has been accepted for publication and a second is being prepared and is awaiting only some further observations of the human rods using longitudinal sectioning to determine fully the nature of the fusing rod outer segments we have seen in these retina. It is important to determine whether the apparent fusing of the human rod outer segments is due to true fusing of separate rod outer segments (which would presumably be a pathological condition) or due to dislocation and folding upon itself of an individual rod outer segment, (which would be more indicative of aging, as

reported to us by John Marshall from his studies).

## 6. MONOCHROMATIC LIGHT EFFECTS ON RETINA

Quails and goldfish (which have high cone retinas) were subjected to monochromatic light at "red" and "blue" wavelengths. Some animals were light adapted, and some dark adapted, and some were kept in "normal" light, and some in continuous dark.

The retinas were processed by prolonged osmication at 40°C according to the method of Pourcho & Bernstein (1975) who found that the amount of osmium accumulation in the outer segments and synaptic regions was increased by light stimulation and decreased by lengthy dark adaptation. We wanted to see if this method could be used to locate differences within outer segments in the differing treatments. We may note in this connection that Grün (1975) and Yakob et al (1977) have reported that the cone outer segments in two different species of fish appear to be divided lengthwise into individual packets or subgroups.

The retinas so-treated crumbled in the processing and a telephone call to Pourcho revealed that this is a hazard of the treatments and many trials may be required for success. While this could well prove a most valuable technique we have for the time being reluctantly put this aside to concentrate on more promising avenues which do not require as much technical development time.

## 7. DEVELOPMENT OF SOFT X-RAY LITHOGRAPHY FOR THE STUDY OF LIVE RETINAL CELLS

We have attempted to develop new tools to be used in our study of retinal structure and of microdamage within the retinal cells. In particular we are attempting to develop a microscopy/spectroscopy which will eventually permit us to examine the light sensitive elements, the rods and cones, in vitro and as a function of real time after light abuse.

It has recently been demonstrated that the lithographic technique which was developed for the manufacture of microscopic electronic devices can now be effectively used in the study of thin biological samples (Spiller et al, 1976a, Feder et al, 1977; McGowan et al, 1977). The technique is a natural extension of contact micrography which was invented some years ago, but which today can potentially find extensive use resulting from 1) the recent development of grainless films (X-ray resists) and 2) extremely intense, highly collimated and monochromatic X-ray sources such as one obtains from a synchrotron electron accelerator. In our studies we have used PMMA (plexiglass) as our film, spun < 2μm thick upon a structureless disk of highly polished silicon. The method is that developed by the group at IBM Yorktown Heights who are assisting us with our studies.

In the previously reported experiments the less than 2 μm thick biological sample was either plated directly upon the resist as in the case of our studies thus far or mounted on a 2 μm thick film of mylar which is partly transparent to the X-rays used and put in close contact with the PMMA. The sample-

resist open-face sandwich was then irradiated (Fig. 6). The amount of X-radiation which reaches and damages the resist reflects both the density of a particular atom in the sample and the elemental makeup of the absorbing cell. As a result, by changing the wavelength of the bombarding X-rays one can expect, and does see, a change in the absorption pattern depending on the composition of the cell under study.

Once irradiated the cells are removed with ethylalcohol on a Q-tip. The irradiated resist is then developed with a mixture of IPA and MIBK using the techniques which have previously been described (Spiller et al, 1976b). The rate of dissolution of the damaged and partially decomposed resist depends critically upon the amount of energy absorbed in the resists. Upon the completion of development, one has a relief replica, reflecting the differential absorption of the specimen. The plastic replica is then metallized and read back with a scanning electron microscope. With soft X-rays ($\lambda \sim 2.5$ nm) the ultimate resolution of the technique is near 5 nm which is approximately the range of secondary electrons produced in the X-ray resist by X-ray bombardment. For lower energy X-rays in the vicinity of the carbon K-edge (4.48 nm), the resolution is just the diffraction limit.

In the first of three publications reporting on the use of X-ray replication in biological studies, diatoms were replicated with 4.48 nm and with the radiation from the DESY Synchrotron radiation source in Hamburg, Germany, where an energy continuum between 4.5 and 2.5 nm was used. In these studies

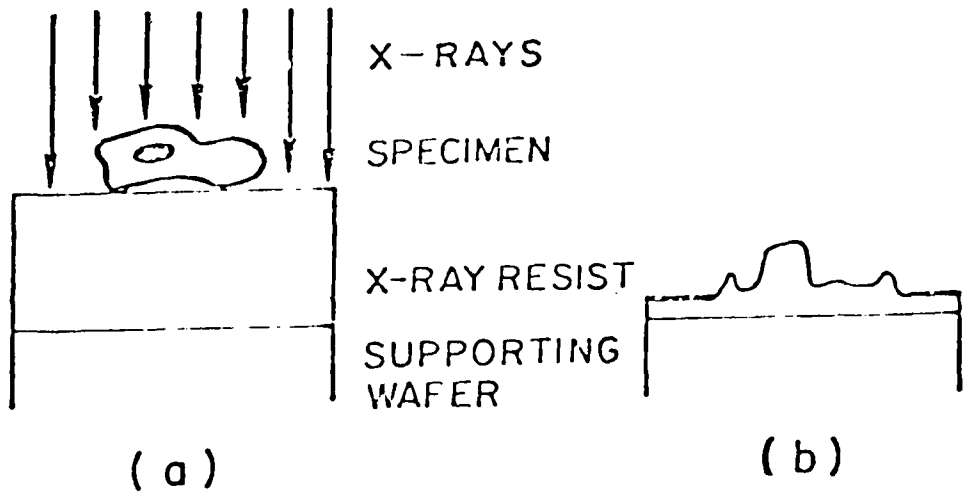


Fig. 6. (a) X-ray irradiation of the cell-PMMA open faced sandwich.

(b) Replica of cell in PMMA after development.

resolutions approached 100 nm, approximately one order of magnitude better than the work reported by Ehrenberg and White in 1959 using Al K$\alpha$ radiation, and Kodak emulsior 22440 as the film.

In a yet to be printed second report (Feder et al, 1977) the chromosome from the salivary gland of the fruit fly Drosophila was replicated with 4.48 nm Carbon K radiation with a resolution approaching 25 nm. Also in that same report the uranium stained pigment epithelial cell of frog retina was replicated using the 2.5-4.5 nm continuum radiation from the DESY synchrotron radiation source. In this case the resolution was better than 10 nm approaching the upper limit which is expected for the technique.

In a third publication which has grown out of our study we have chosen to use heart of chick embryo rather than retinal cells for our tests. They were incubated and freeze-dried directly upon the PMMA disk. Some were replicated with Carbon K radiation at the IBM Laboratories while others were irradiated with the 2.5 to 4.5 nm continuum from the DESY Synchrotron radiation source. In our study (refer to appendix) for draft) we have made the first systematic study of the microscopic and crude spectroscopic potential of soft X-ray replication. Here we have a resolution better than 20 nm and for the same cell demonstrate a marked difference in the prominence of the structures seen when replication is made at the different wavelengths.

Just as we can see bones in the hand through the flesh with harder X-rays we can now see quite clearly the internal structure of the cell with soft X-rays. Because the amount of

structure one observes is extensive and variable with the wavelength of the radiation one wonders at first whether or not the ordering one sees is not to a large extent an artifact caused by the large angle at which the SEM stage is set in order to highlight the hills and valleys formed in the PMMA as a result of the radiation damage. In Fig. 7 we show quite clearly the nuclear region of a cell replicated with 4.48 nm radiation. The replica is observed at a number of different angles and orientations. In this sequence one can track the various bumps and valleys thus removing the doubt that what we see at one angle is purely an accident of tilt angle.

In Fig. 8(a and b) we show the details of yet another replication made with 4.48 nm radiation and viewed with the UWO Hitachi HHS-2. In this case we detected the secondary electrons emitted by the sample. In Fig. 9 we show the micrograph of another cell replica studied with the SEM using the electron backscatter mode of operation. In this later case the resolution is substantially inferior yet some details are much more pronounced.*

We have already made our first attempt to replicate live cells in specially prepared hermetically sealed chambers in which living mouse "L" cells brought from Canada to Germany were incubated. Our X-ray source was the DESY Synchrotron. Although we were able to produce replications of

* It is unfortunate that most of the electron micrographs of the heart of chick embryo cells, mouse "L" and retinal cells were lost when the papers of J.Wm. McGowan were stolen this Spring in Rome. This has necessitated a re-scan of many of the PMMA disks irradiated last Fall. Some of the most informative disks have unfortunately been destroyed and were not available for re-study.

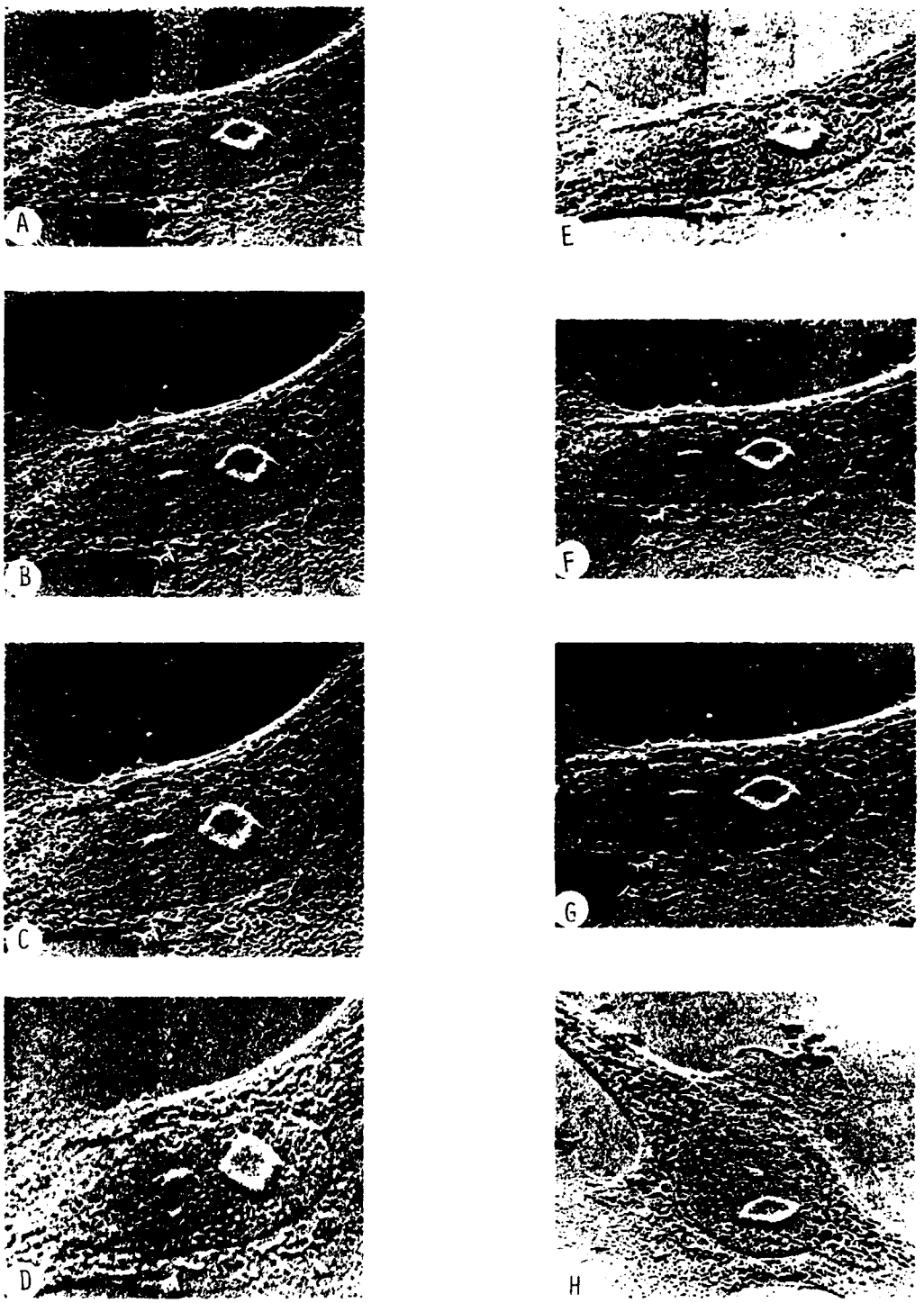


Fig. 7. Replica of the nuclear region of the heart of chick embryo cell in which the nucleus, one large nucleolus and considerable other structure can be identified. The eight replications depicted (all magnifications 4.8 K but for (b) show the cell mounted at different angles of tilt and at different angles of rotation on the SEM stage. In the first four the SEM table is tilted at a) $60^{o}$; b) $50^{o}$; c) $40^{o}$; d) $30^{o}$ with the electron suppresser off: e) is as in (a) but with suppresser off; f) rotated $12^{o}$ and tilted to $60^{o}$; g) as in (f) but with the magnification increased to 5.4K; g) rotated $80^{o}$ and tilted $60^{o}$.

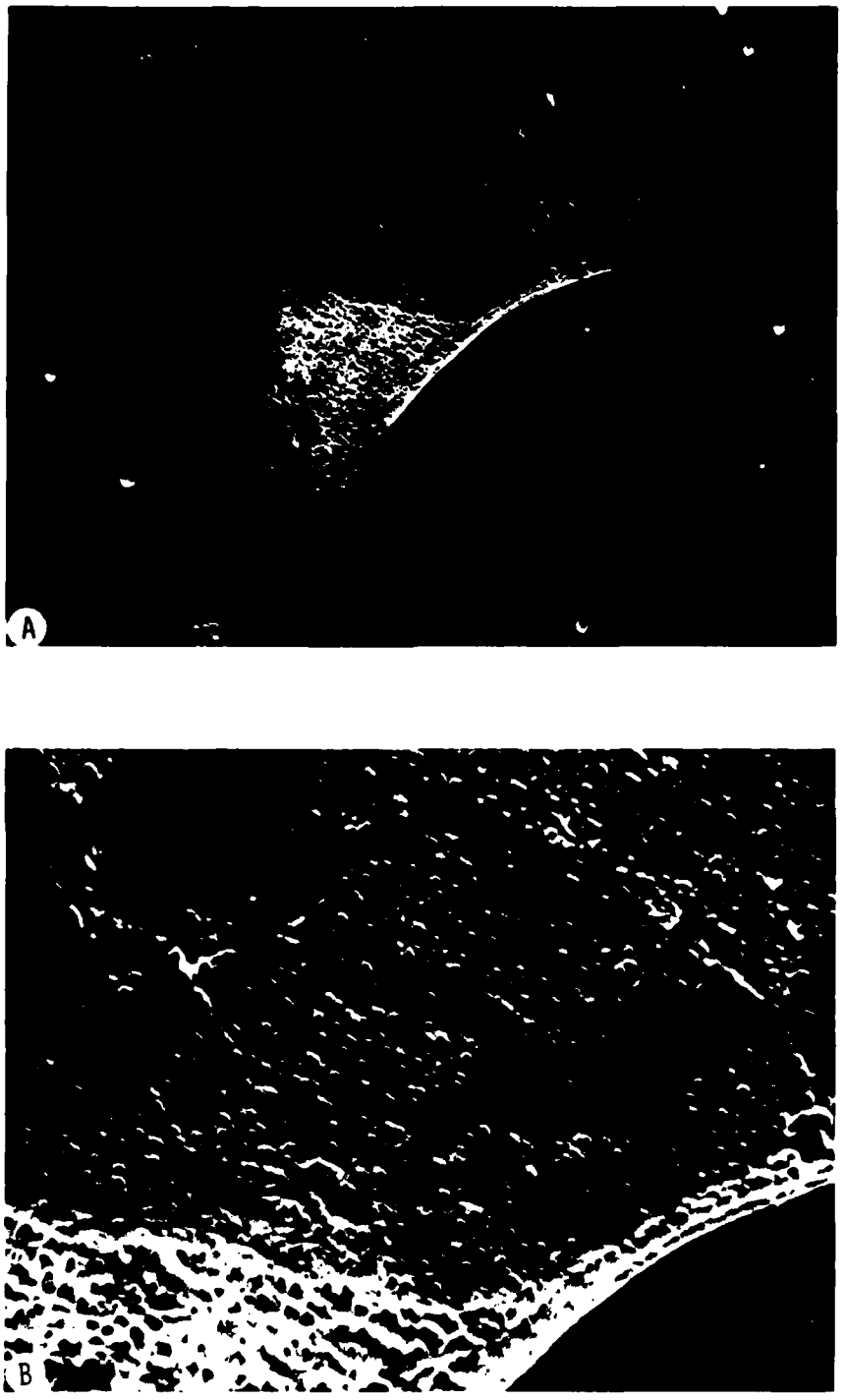


Fig. 8. a) a 4.48 nm replica of heart of chick embryo cell showing clearly a good margin at the nucleus, and a double nucleolus within the nucleus. Bundles of mitochondria are clearly in the pseudopodia. Magnification 3K.
b) nuclear region taken with a magnification of 10K.

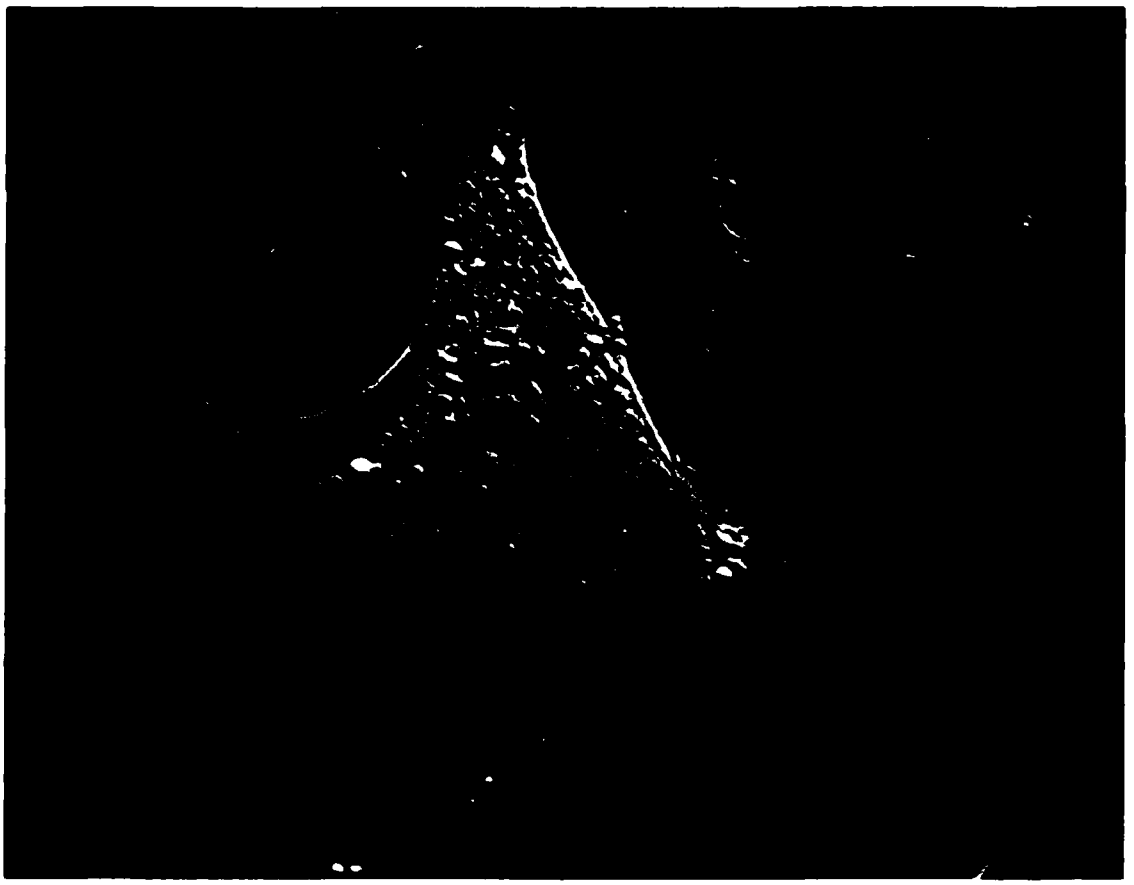

Fig. 9. A 4.48 nm replica of heart of chick embryo cell where the nucleus and two nucleoli are visible. One can clearly identify lamellapodia on the three pseudopodia extending from the nuclear region. Large droplet structures (presumably lipid droplets) and the microspikes at the periphery of the cell wall can be easily identified. There is definite evidence of mitochondria in the pseudopodia. Magnification 3K.

the living cells in vitro they are not clear, suggesting that in the 4 to 8 minutes it took to make the replica, the cells moved. That such movement can account for our findings has been confirmed by time-lapse photographs made by Prof. R.C. Buck of "L" cells using the light microscope to record the motion as a function of time and crudely as a function of temperature.

There are a number of experiments which are presently underway designed to further test 1) the effectiveness of low energy x-ray replication as a spectroscopy, 2) the usefulness of these techniques in studies of live cells, and 3) the possibility of making carbon replicas of our plastic replicas to be read back with the TEM, and most important for this program 4) the applicability of the newly developing techniques to high resolution studies of freeze dried, fixed and live retinal material. In the next paragraphs we outline these programs.

Two spectroscopic studies are presently underway. In the first at IBM we are using the bacterium Bacillus subtilis (BS) as our test cell since Dr. Beveridge from the Bacteriology Department of our University has been studying the BS cell wall as a function of metal takeup. As our X-ray source, we are using various K and L X-ray lines in the IBM X-ray machine. However, we have just completed the development of our own tunable source here at UWO. The lines chosen are just above and just below the K,L or M onsets of absorption for the elements of interest to us. By comparing the two micrographs we will further test whether or not specific spectroscopic identification can

be made of the metal substituted in the < 15.0 nm wide cell wall. At the same time we are again studying our test cell "heart of chick embryo" in an attempt to further test the method.

At DESY, Hamburg, preparations are now underway to crudely tune the synchrotron radiation with a reflection grating while at the same time the X-rays are concentrated onto a smaller surface area. The attempts at focussing are being made to increase the radiation dose and to once again diminish the time necessary to make a replica.

During the June run at DESY it is our plan to once again try to replicate live cells, again heart of chick embryo cells since we have amassed considerable data on this system. We also plan to replicate live rods and cones teased from frog retina. This later study is being done in conjunction with Dr. John Marshall and his associates at the Institute of Ophthalmology London, England.

At the Facultés Universitaires, Namur, Belgium, we are presently attempting to make carbon replicas of our PMMA replicas of heart of chick embryo. This is done to facilitate TEM reading of the X-ray replica in order to again improve resolution, to minimize the effect of the tilt angle of the SEM stage, and to eliminate the serious problem associated with the time dependent SEM electron beam degrading of the plastic replica.

Finally, it is also our plan in the June X-ray run at DESY to examine some of the lateral sections of damaged and normal retina now under study in our laboratory. At the same

time and for comparison we plan to replicate isolated rod disks which again have been prepared by the group in London, England.

During the next period our program of X-ray replication is to receive partial assistance from the Medical Research Council, Canada, which is supplying financial support for a post-doctoral fellow who will work in this area.

8. PAPERS AND COLLOQUIA DELIVERED

"Scanning Electron Microscopic Studies of Normal and Lased Rabbit Retina and Pigment Epithelium" - a paper presented by Dr. J.A. Medeiros to the Canadian Ophthalmological Society Meeting, Quebec City, June, 1976

"SEM of Normal and Lased Rabbit Retina and Pigment Epithelium" - colloquium presented by Dr. Bessie Borwein to the Dept. of Ophthalmology, Ben Gurion University, Beersheba, Israel, July, 1976

"The Role of Cones in the Visual System" given at the Centre d 'Analysis et de Researches Interdisciplinaire de Namur, Facultes Universitaires de Namur, Belgium. This talk was also given at the U. Kaiserslautern, West Germany and at the Dept. of Physiology, University of Toronto, J.Wm. McGowan

"SEM of Normal and Lased Rabbit Pigment Epithelium", at Ann Arbor, Michigan, October, 1976, to Midwest Anatomists Association, Dr. B. Borwein

"Mammalian Retinal Cone Structure" given to the Workshop on Retinal Cones & Colour Vision at U.W.O., February , 1977. Dr. B. Borwein

"Renewal and Turnover in the Vertebrate Retina", an invited lecture given March, 1977 at the University of Waterloo School of Optometry, Dr. B. Borwein

"Cone Shape and Colour Vision" given to the Workshop on Retinal Cones & Colour Vision, U.W.O., February , 1977, Dr. J.A. Medeiros.

"Synchrotron Radiation for Research and Industry", U. Louvain-la-neuv, Departement de Physique, Brussels, April, 1977

J.Wm. McGowan

9. PAPERS PUBLISHED

1. B. Borwein, M. Sanwal, J.A. Medeiros and J.Wm. McGowan, 1976; Can. J. Ophthal., 11(4): 309-322, Scanning Electron Microscopy of Normal & Lased Rabbit Retina
2. B. Borwein, M. Sanwal, J.A. Medeiros and J.Wm. McGowan, 1977; Invest. Ophthal. (in press), Scanning Electron Microscopy of Normal and Lased Rabbit Pigment Epithelium.
3. B. Borwein, J.A. Medeiros and J.Wm. McGowan, 1977, Invest. Ophthal. (in press), Fusing Rod Outer Segments from an eye enucleated for choroidal melanoma.

In preparation

1. B. Borwein, So Called Normal Areas of the Retina in an eye enucleated for choroidal melanoma (almost complete).
2. J.A. Medeiros, et al, Application of Optical Transform Techniques to Laser Irradiation of the Eye (Appendix "A").
3. J.Wm. McGowan, et al, Towards the Development of High Resolution X-Ray Microscopy/Spectroscopy of Cells (Appendix "B").

## 10. ORGANIZATION OF INTER-UNIVERSITY WORKSHOP GROUP

During the past year we have initiated the formation of a workshop group of researchers interested in various aspects of vision from a number of local universities. The initial meeting of the group was held at The University of Western Ontario on February 8, 1977. Subsequent meetings have been held at York University (Toronto) and at the School of Optometry, University of Waterloo. Members of the group include researchers in visual psychophysics (W.D. Wright, G. Wyszeki, P. Kaiser), electrophysiologists (R. Beauchamp) and biochemists (E. Abrahamson). In addition to the stimulating exchange of ideas and interpretations of current visual research a number of experiments and some proposed joint investigations have been inspired by the meetings of this workshop group.

## 11. WORKING GROUP "CHRONIC LIGHT DAMAGE TO THE EYE"

Dr. Bessie Borwein attended the meeting of the working group on "Chronic Light Damage to the Eye" on the invitation of the Chairman, Dr. T. Lawwill. This meeting of 19 people was sponsored by the National Academy of Science Committee on Vision and met in Sarasota for the day on April 23rd. The Deans of Medicine and of Science at U.W.O. provided the necessary funds for this, and also for the ARVO meeting. Dr. Borwein's presentation of some of the work of the Laser Eye Group went well and was met with considerable interest at the time, and subsequently. The ARVO meeting provided an excellent opportunity to keep in touch with major developments in retinal research and to discuss some of our work.

## 12. VISITS OF DAVID H. SLINEY AND JOHN MARSHALL

a. We particularly appreciated the visit in April, 1976 of David H. Sliney of the Laser-Microwave Division, U.S. Army Environmental Hygiene Division, Aberdeen Proving Ground. Our discussions with him on questions of laser safety and the research problems of defining exposure standards were both important and stimulating. His talks were of interest to the local medical community and other researchers in the University as well as the Canadian Defence and Civil Institute of Environmental Medicine (DCIEM) who share his interest in laser hazards and protection for military personnel. Mrs. Sharon McFadden of DCIEM visited concurrently for the purpose of discussing these interests with Mr. Sliney.

b. During a visit in May, 1977 Dr. John Marshall of the Institute of Ophthalmology, London, demonstrated a number of interesting and potentially extremely useful techniques for histological examination of the retina in both normal and light damaged conditions. Dr. Marshall's own very important studies have been directly applicable to the concerns of our own research. He has observed, using light microscopy of transversely sectioned retina, the existence of spur-like extensions at the distal tips of the retinal cones to the pigment epithelium. This same feature, the existence of which has not yet been revealed in the literature, has been seen in our TEM observations of transversely sectioned retina discussed above.

Dr. Marshall has confirmed in private communications during his visit that he also observed a taper in the central cones of the human retina. His visit was most stimulating and we were able to discuss many aspects of our work.

An extension of the developments outlined in this report will be the undertaking of experimental observations of unfixed "live" or "wet" retinal cones by the X-ray lithographic technique in conjunction with Dr. John Marshall at the DESY Synchrotron facility in Hamburg, Germany.

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# APPLICATION OF OPTICAL TRANSFORM TECHNIQUES TO LASER IRRADIATION OF THE EYE

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## Introduction

In certain applications it is necessary to expose an eye to a number of separate irradiations with a laser beam. For example in experiments designed to determine the threshold for laser-induced damage to the retina, the usual procedure consists of subjecting a test eye to many separate exposures at a range of irradiance levels spanning the threshold value that produces an effect meeting some minimum damage criterion. The accuracy with which the effective dose which results in an observable effect 50% of the time ($ED_{50}$ level) can be determined depends on the accumulation of statistically significant numbers of exposure trials. In addition to the time consuming nature of this procedure, where each exposure must be logged and recorded separately, one must be concerned that the physiological state of the test eye may alter over the course of a given run (one to two hours) and that later exposures may not produce exactly the same effect as earlier ones. In other applications, as in clinical treatments, it

may be required to expose the human retina to a large number of separate exposures (as in "panretinal bombing"). This can be a time consuming and exhausting procedure for patient and ophthalmologist alike.

Some of the difficulties associated with repeated application of many separate exposures can be alleviated by taking advantage of the coherent nature of laser light and some basic optical principles. This paper describes some techniques we have used in our laboratory for retinal damage studies whereby an incident laser beam is modified by passage through an appropriate diffracting screen to give any arbitrarily desired irradiance pattern at the retina of a test eye. These include patterns with many separate spots with a range of intensities delivered by one exposure.

## Diffraction of Light and the Far-Field Patterns at the Focus of a Lens

The diffraction of light by a screen with an arbitrary transmittance function is, in principle, given by the solution of Maxwell's equations for the electromagnetic fields subject to the imposition of the boundary conditions at the screen. In practice, this direct approach can only be carried out for problems with particularly simple geometry. The scalar theory of diffraction, at the cost of neglecting (usually) minor polarization effects, affords considerable simplification in the description of the electromagnetic wave scattering. The formulation is based in essence on Huygen's

principle of re-radiation; each point of a diffracting screen is treated as a new source of radiation proportional to the field strength incident on it and the transmittance of the screen at that point. In effect, the pattern of illumination at any point beyond the screen is the result of the combining or superposition of the fields from each point on the screen.

The classic example illustrating the principles involved is Young's two-slit experiment. In Figure 1, monochromatic plane waves of wavelength $\lambda$ (and wavevector $k = 2\pi n/\lambda$, where n is the refractive index of the medium ) is incident on two small slits separated by a distance d. Each point in the slits can be thought of as a source of spherical waves of amplitude

$$\psi = af_s \frac{\exp(-ikr)}{r} \qquad (1)$$

at a distance r from each point source, where a is the amplitude of the radiation incident at the slit and $f_s$ is the transmittance of the screen (varying between zero for opaque and 1.0 for transparent regions). At the intersection of the wavefronts as pictured in the figure, the field intensities add constructively giving rise to interference fringes in a viewing plane beyond the diffracting screen. The intensity maximum on the axis perpendicular to the plane of the two slits and equidistant between them result from the addition of wavefronts which are the same distance from each slit. These wavefronts arrive with the same phase delay, kr, from each source. The addition of wavefronts differing in phase by an integral

multiple of $2\pi$ give rise to successively higher order interference fringes on points off axis in the viewing plane.

When the observation plane is far from the diffracting screen as compared to the slit separation and the slit separation itself is large compared to the optical wavelength, the angular deviation of successive interference fringes can be found through simple geometric construction to be given by

$$\sin\theta = m\lambda/d \qquad (2)$$

where the order of the interference term is $m = 0, \pm 1, \pm 2, \ldots$

This is just the usual grating equation giving the position of the light diffracted by an array of many periodically placed slits (with spacing d between transmitting regions). The field intensity at any point beyond a diffracting screen is obtained by integrating the contribution from each point on the screen. The resulting field amplitude depends on the phase delay in the wavefronts arriving at the observation point due to each source on the diffracting screen. For objects more complicated than a simple array of slits, the phase delay introduced by each point on the diffracting screen will be more complicated to sum and will give a more complicated diffraction pattern.

If the contribution to the phase delay in waves arriving at a given observational point from a diffracting screen is a linear function of the coordinates in the plane of the screen (a condition which obtains for a viewing plane far from the diffracting screen where the outgoing spherical waves are well approximated by plane waves) the diffraction is

said to be of the Fraunhofer type. When this approximation is not valid and the phase delay introduced by each point in the diffracting screen is a quadratic function of the co-ordinates of the aperture, the diffraction is of the Fresnel type. Fresnel diffraction is much more complicated than Fraunhofer diffraction but fortunately it is of little more than academic interest and does not concern us here.

The criterion of observing diffraction at a great distance from the diffracting screen, where the outgoing waves are planar, is just the condition found in the focal plane of an optical system. The far-field diffraction pattern at the focal plane of a lens is the Fraunhofer diffraction pattern of the object in front of the lens. An important principle of optics is that the Fraunhofer diffraction pattern of an object is the Fourier transform of the transmittance function of that object (c.f. Born and Wolf, 1965; Lipson and Lipson, 1968). It can be shown (c.f. Shulman, 1970) that if the area of consideration in the input (diffracting) plane and the back focal plane of a lens of focal length f are respectively restricted to domains with coordinates given by

$$(x_1^2 + y_1^2)^{\frac{1}{2}} < f/5$$

and (3)

$$(x^2 + y^2)^{\frac{1}{2}} \quad 0.14f \ ,$$

then the light amplitude distribution A'(x,y) in the focal plane of the lens is given by

$$A'(x,y) = \frac{-i\exp[ikR(x,y)]}{\lambda(f+d)} F(u,v) \qquad (4)$$

where

$$u = x/f, \quad v = y/f, \tag{5}$$

$$R(x,y) = \frac{f^2 + df + x^2 + y^2}{(f^2 + x^2 + y^2)^{1/2}}, \tag{6}$$

and $$F(u,v) = \int\int A(x_1,y_1) \exp[-2\pi i(ux_1 + vy_1)]\, dx_1\, dy_1 . \tag{7}$$

F(u,v) is just the Fourier transform (two dimensional) of the light amplitude distribution $A(x_1,y_1)$ a distance d in front of the lens. The physically measurable quantity in the transform plane is the light intensity which is the square of the amplitude distribution:

$$|A'(x,y)|^2 = \frac{1}{\lambda^2(f+d)^2} |F(u,v)|^2 \tag{8}$$

The computation of the Fourier transform of many simple diffracting objects is readily evaluated. The diffraction from more complex objects can often be found as a combination of these simple cases where the linearity of the Fourier transform can be exploited (the transform of a product or sum is the product or sum of the individual transforms). In particular it is important to note that the transform of a Gaussian function is itself a Gaussian. Thus, in passing a laser beam with a gaussian profile through a diffracting object the light intensity in the transform plane will be the convolution of the diffracting object and a gaussian. An important special case for the diffracting object is that of a transmission function consisting of a periodic array of alternately transmitting and opaque bands (diffraction grating).

## The use of Gratings for retinal irradiation

A Ronchi ruling is a square wave grating with alternating opaque and transparent regions of equal width. Such a square wave train, alternating between the amplitudes 0 and 1 with an angular frequency $\omega$ ($2\pi$ times the grating frequency), can be synthesized as the infinite Fourier series

$$f(y_1) = \tfrac{1}{2}\left(1 + \frac{4}{\pi}\cos\,\omega y_1 - \frac{4}{3\pi}\cos\,3\omega y_1 + \frac{4}{5\pi}\cos\,5\omega y_1 - \ldots\right) \quad (9)$$

which is a series consisting of a DC term ($\tfrac{1}{2}$) representing the mean amplitude of the square wave and the alternate addition and subtraction of the cosine of the odd harmonics of the fundamental angular frequency of the periodic diffracting object. Writing the cosine as its component complex exponentials, this is:

$$f(y_1) = \tfrac{1}{2} + \frac{1}{\pi}e^{i\omega y_1} + \frac{1}{\pi}e^{-i\omega y_1} - \frac{1}{3\pi}e^{i3\omega y_1} - \frac{1}{3\pi}e^{-i3\omega y_1} + \ldots \quad (10)$$

The Fourier transform of each of these terms gives a point in the inverse frequency space (transform plane of the lens). Thus the optical transform of a Ronchi ruling will consist of points in the focal plane of the lens corresponding to the associated frequencies; there will be a DC or zero order term of amplitude $\tfrac{1}{2}$, and the $\pm$ odd harmonics of $\omega$ (namely $[2n+1]\omega$) of amplitude $(1/[2n+1]\pi)$. The positions of the spectral components in the transform plane are odd multiples of the quantity $y_o = \lambda f\omega/2$ when the grating is placed in the front focal plane of the lens. The scattering geometry and optical spectrum of a Ronchi ruling is shown in Figure 3.

Figure 4 illustrates the result of exposing a rabbit retina to a laser beam passed through a Ronchi ruling placed in front of the eye. A number of single exposures (without a grating) are visible on the retina; the linear sequence (arrow) is that made by the transformed beam. Only three lesions, corresponding to the zeroth and first order (± 1) terms are visible in the sequence. As a method of threshold determination, such a grating would not be suitable since most of the energy goes into the zeroth and first orders. The higher diffraction orders, where the energy decreases more gradually are well below threshold. Using high enough energies to bring the higher orders above threshold would be precluded here since the low orders would be so intense that considerable damage would be done to the retina.

It is possible to remove the lowest orders from the beam by using the interchangeability property of the Fourier transform whereby the transform of the transform is the original function. By passing the laser beam through a grating followed by a lens, the low orders can be blocked in the transform plane with a suitable obstacle (spatial filtering). It is then only necessary to position the test eye so that the image of the now modified grating is in front of the pupil. The eye will then focus the transform of the modified grating (as if the physically modified grating were actually in place at the location of the image formed by the lens of the spatial filter). One can then use much higher intensities and the higher orders will appear on the retina with the blocked orders missing.

One may also extend the diffraction pattern in two dimensions using two crossed diffraction gratings. The resulting pattern in the transform plane then consists of a matrix of regularly positioned points, the positions of the rows and columns of which are determined by the grating frequency of the separate rulings. Figure 5 is a photograph of the retina of a monkey (macca fasicularus) in which a laser beam was passed through two crossed rulings and the resulting pattern is indicated by the arrows. Note that the spacing of the lesions for a given wavelength, grating frequency and positioning in front of the eye is a measure of the focal length of the eye at the time of the exposure.

The optical transform technique can be made useful for threshold determination by the use of other diffracting screens. A very interesting case is that of a sinusoidal grating. The Fourier transform of a grating made up of a sinusiodal modulation of its transmission will consist of only the zeroth and the first order since the higher frequency harmonics are not present in the original object as is the case of the square wave grating. However, the result is very different if one uses a grating in which the sinusoidal modulations are of transmitted phase, as in a transparent grating of modulated thickness. For such a phase grating in which the sinusoidal modulation is transmitted phase is m radians for the peak to peak excursion, the peak intensity of the qth order is proportional to $(J_q(m/2)^2$ where $J_q(m/2)$ is the Bessel function of the first kind of order q

(Goodman, 1968).

Two examples of the intensity distribution in the first eight orders for peak-to-peak excursions of the phase delay given by $m/2 = 4$ and $m/2 = 6$ are shown in Fig. 6. As can be seen, the trend is for higher proportion of the diffracted power to appear in higher orders for increasing values of the phase excursion. Note that at the zeros of $J_0(m/2) = 0$ there is no power in the zeroth order (at $m = 4.81$).

Such phase gratings are relatively easy to fabricate. One may make a sinusoidal grating by recording the interference fringes at the intersection of two plane coherent light beams. The spacing of the sinusoidal fringes so produced are given simply by the grating equation, $d = \lambda/\sin\theta$, where $\theta$ is the angle between the two beams. The pattern can be recorded as sinusoidal modulations of optical density on a photographic film.

This sinusoidal amplitude grating can be converted into a sinusoidal phase grating by bleaching the photographic negative (cf. Collier, et al, 1971). In the bleaching process the modulations in optical density in the film are converted into variations in transmitted phase by the occurrence of two processes; the oxidation of the silver grains to a transparent silver salt having a refractive index larger than that of the gelatin of the emulsion and by the tanning of the gelatin near the silver grains in which a larger than normal degree of cross-linking of the gelatin molecules is induced with the consequent result that the processed emulsion has a

greater thickness (or surface relief) at the location of the hardened or tanned gelatin. A recommmended oxidizing agent for bleaching of Kodak 649F emulsions (6 microns thick) is a 6% solution of potassium permanganate; Kodak D-19 developer should be used to develop the emulsion (cf. Collier, et al, 1971). Much higher exposure intensities are necessary for bleached emulsions than for the production of the unbleached gratings. Exposures should be on the order of 1.0 mj/cm$^2$.

The magnitude of the phase excursion can be controlled by sandwiching the emulsion with a layer of oil between two optical flats. By the use of different oils with different refractive indices one can make the thickness excursion on the emulsion correspond to the desired phase excursion.

The production of Abitrany irradiance patterns

While most of the discussion has focused on the use of gratings as the diffracting screen, other patterns can be used as well, of course. For the production of any arbitrarily desired pattern on the retina of the eye, it is only necessary to place the Fourier transform of the desired pattern in front of the test eye.

The required transform pattern can be made by photographing and reducing a black and white drawing of the desired pattern or by drilling small holes in an opaque screen. However, one is very restricted in the kinds of patterns that can be generated by this method since such a screen contains only amplitude variations. The phase of the Fourier transform is not recorded by this process. One is thus restricted to generating patterns with real Fourier transforms and this

limits one to centrally symmetric patterns with relatively large central order. The Fourier transform of an even function (centrally symmetric) is real. One must then remove the ambiguity of whether the transform is positive or negative (since one can only observe the square of the transform). The transform can be made all positive if there is a heavy center of symmetry in which the phase information for the higher orders are simply recorded as small modulations of the larger, all positive central component.

However, with only one additional step in the recording process, it is possible to produce any desired illumination pattern in the transform plane of a lens. To record all the information in a Fourier transform one needs to encode both the amplitude and phase information; while this cannot be done by conventional photography this is precisely what is done in holography. There a coherent reference wave is combined with the radiation scattered from the object to be recorded. A high resolution film is used to record the interference fringes resulting from the superposition of the two wavefronts. The full optical information available from an object, including the phase information, is recorded as modulations of the reference wave. After development, illumination of the interference pattern with the reference wave generates the waveform originally produced by the recorded object.

This is exactly the same principle used to record the sinusoidal interference fringes in the phase gratings. The beam interfering with the original reference beam is generated

when the processed phase grating is illuminated with the original monochromatic radiation. In the holographic extension of this technique one need only combine a reference beam at the recording plane with the Fourier transform of the pattern desired on the retina. The recording and utilization geometry is illustrated in Fig. 7. While for holograms it is not ncessary to bleach the photographic plate produced, higher diffraction efficiencies are obtainable with bleached emulsions and more satisfactory results can be thus obtained when the eye is illuminated with the test laser beam.

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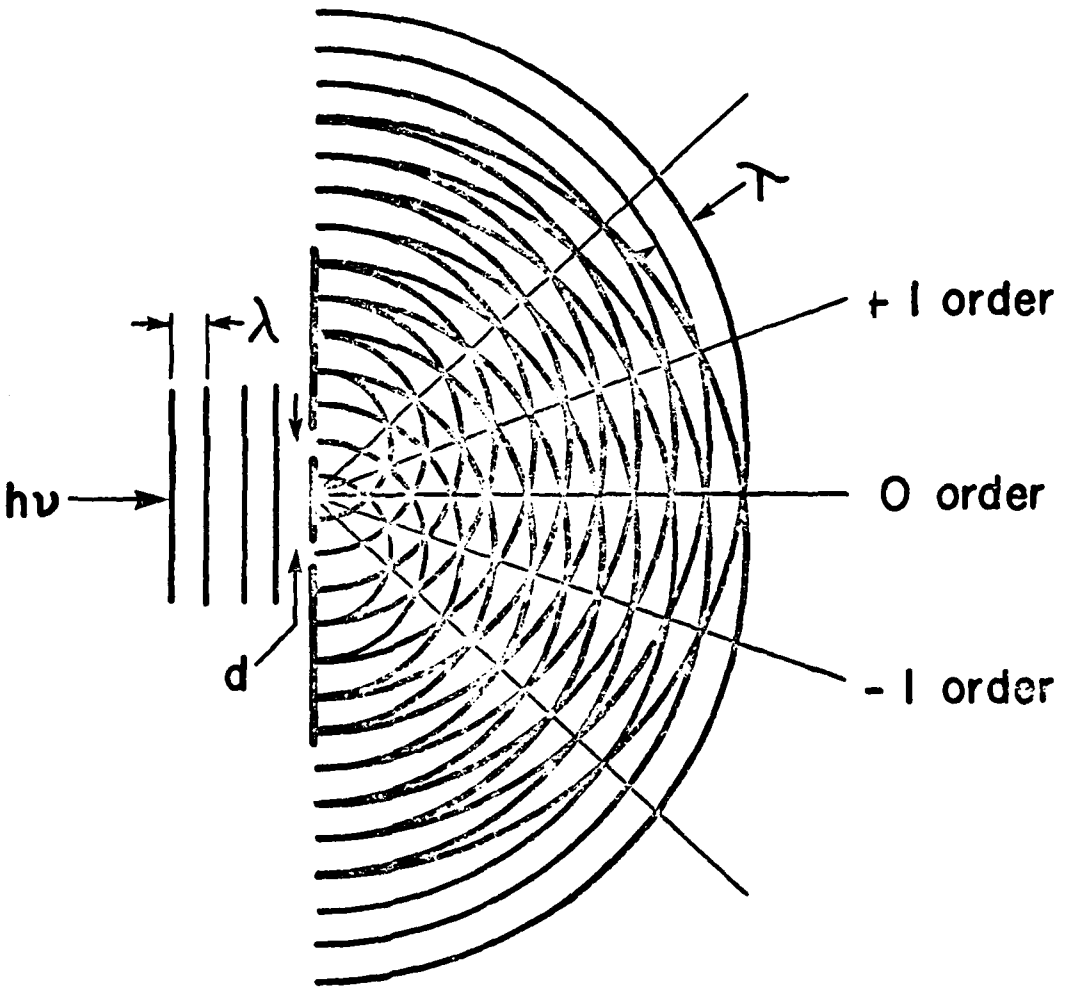
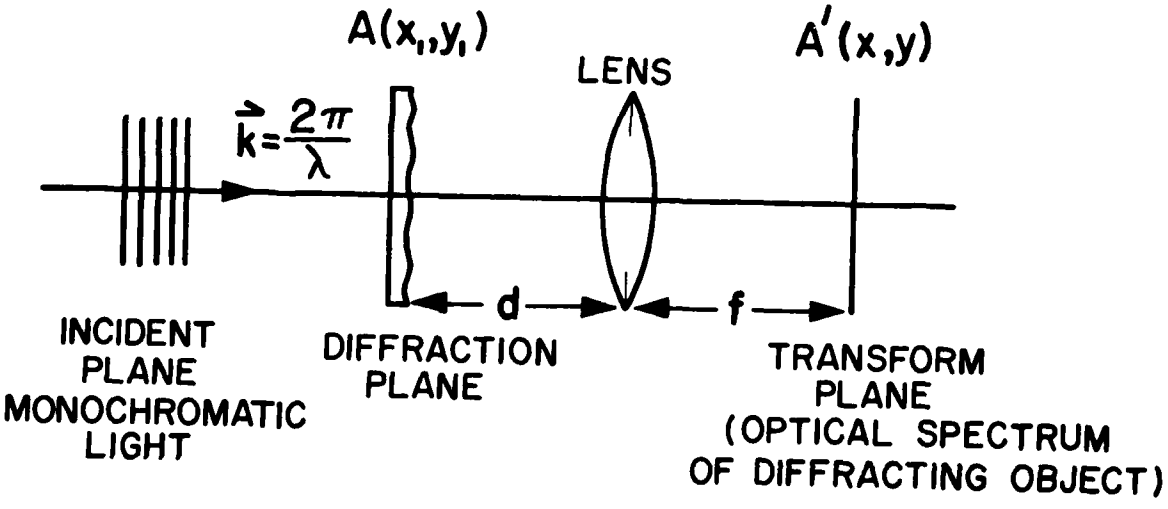



Fig. 1. Young's two slit experiment as the interference of Huygen's wavelets re-radiated at the screen.




$$|A'(x,y)|^2 = \frac{|F(u,v)|^2}{\lambda^2(d+f)^2}$$

F(u,v) IS THE FOURIER TRANSFORM OF $A(x_1,y_1)$

Fig. 2. Showing the relationship between the diffraction plane and transform plane of a lens. The intensity distribution in the focal plane of a lens is the Fourier transform of the distribuiton in the diffracting plane.

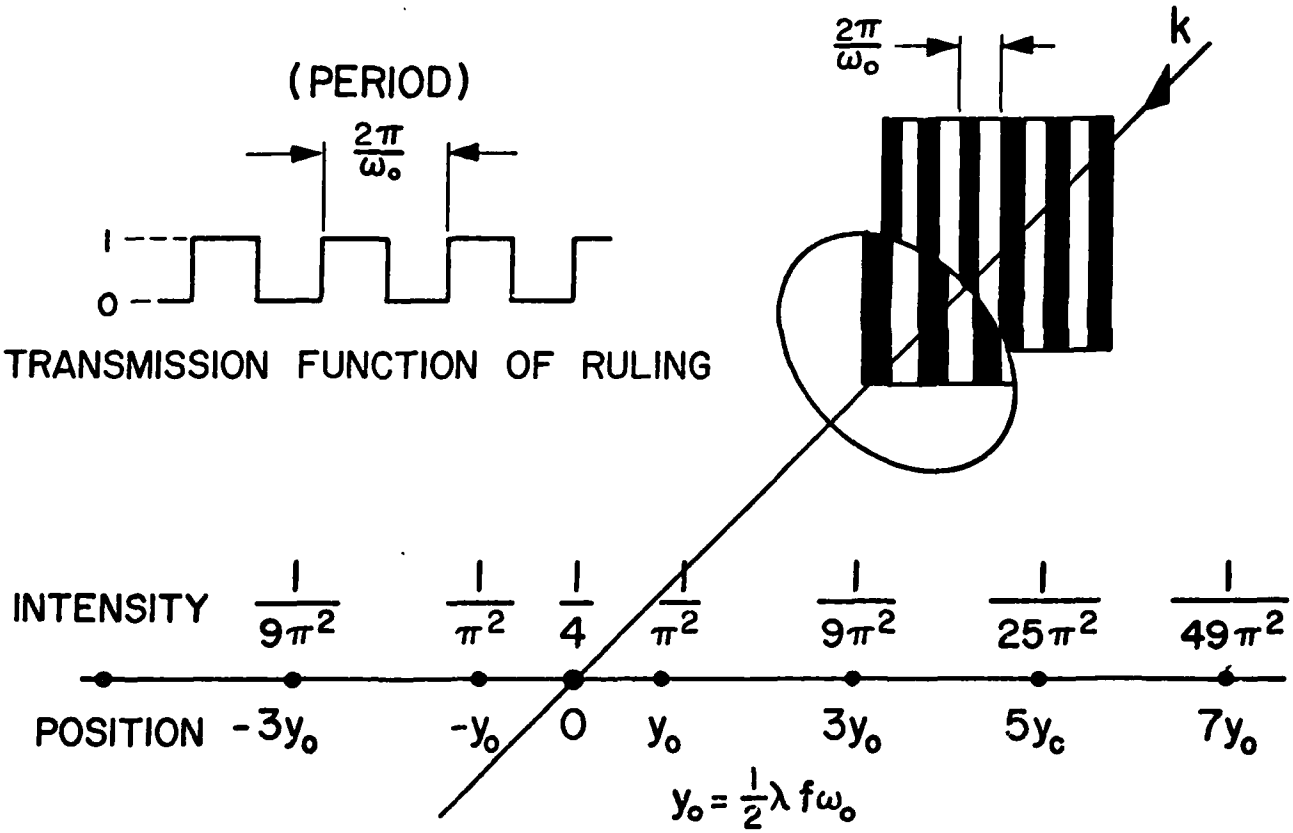



Fig. 3. Scattering geometry and optical spectrum of a Ronchi ruling.

Fig. 4. Fundus photograph of a rabbit retina taken within a few minutes of exposure to laser irradiations including one through a Ronchi ruling. The three spots in a line (adjacent to the large dark lesion) correspond to the bright zero - order and the two smaller first-order components. Higher components are below ophthalmoscopic visibility threshold.

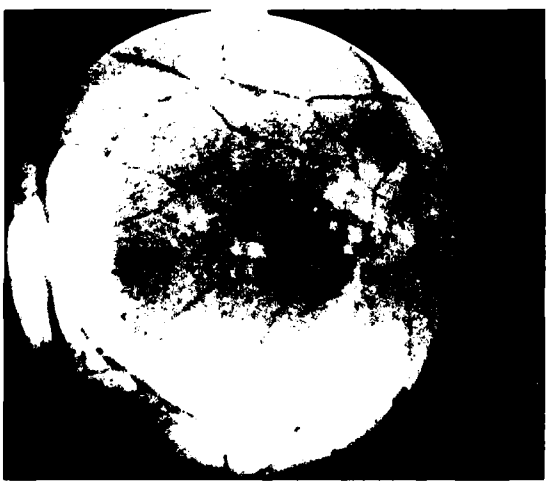


Fig. 5. Fundus photograph of a monkey retina in which crossed gratings have been used to produce pattern of laser irradiations arrayed in the form of a matrix. The zero-order as well as the first order terms in the x and y dimensions are easily visible.

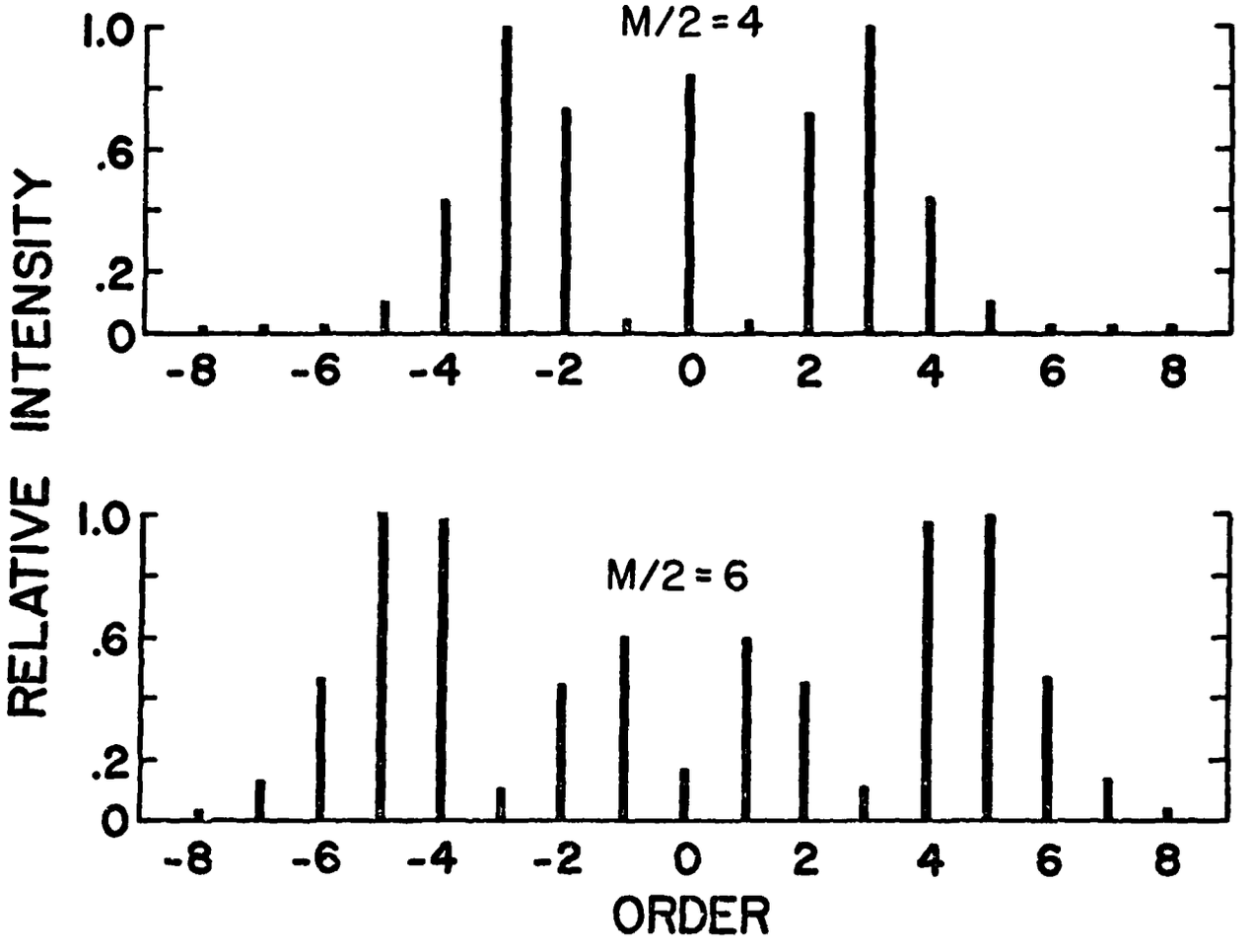



Fig. 6. Two examples of the relative intensity distribution computed for the first eight orders of a phase grating with peak-to-peak phase excursions of m radians.

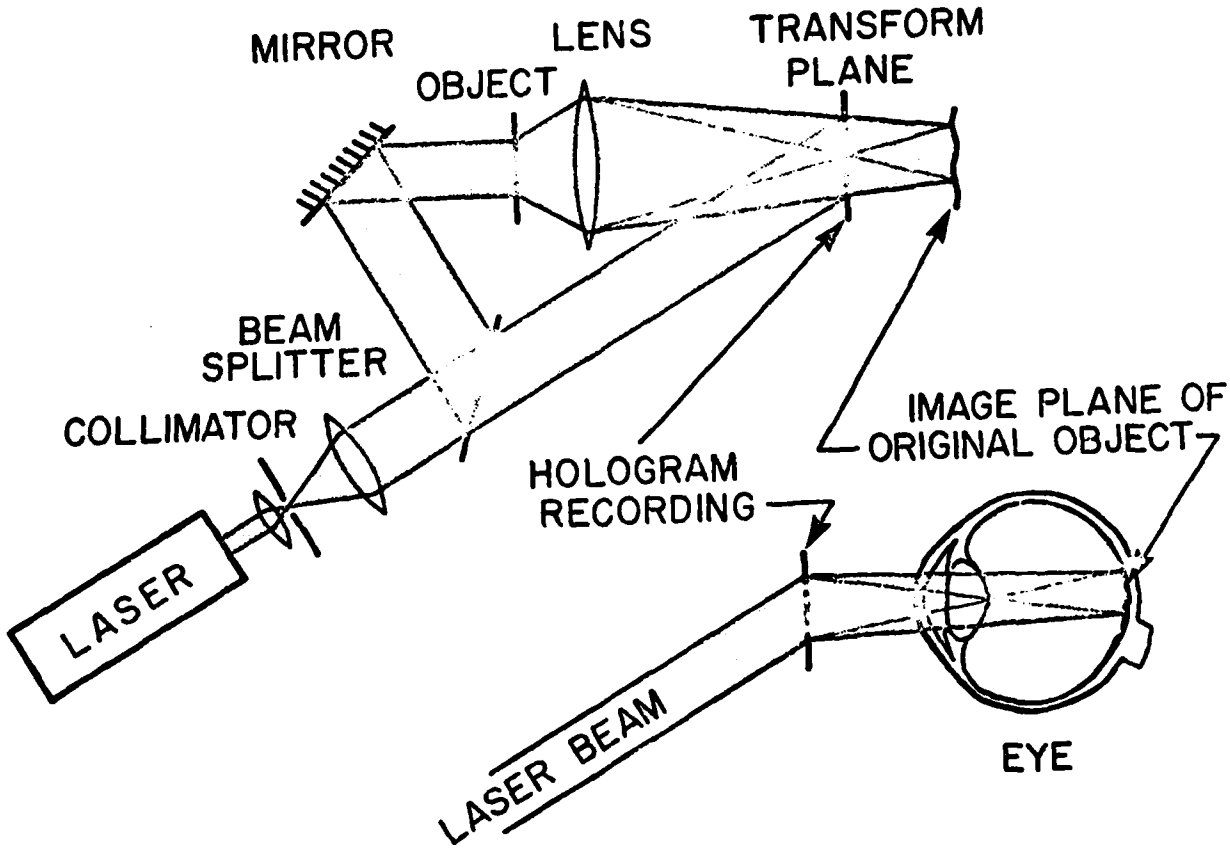



Fig. 7. Geometry for forming and using holographic phase object to produce arbitrary irradiance pattern at retina.

APPENDIX "B"
(submitted to Nature)

# TOWARDS THE DEVELOPMENT OF HIGH RESOLUTION X-RAY SPECTROSCOPY/MICROSCOPY OF CELLS

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It has recently been demonstrated that lithographic techniques developed principally for the manufacture of microscopic electronic devices can be used for high resolution studies of thin stained and unstained biological samples.[1,2] In this report we examine the replicas made of critically point dried heart of chick embryo cells with soft X-ray radiation of different wavelengths and investigate for the first time the usefulness of X-ray replication techniques as a spectroscopic as well as a microscopic tool. We also further examine the potential of the method for studying unstained, fixed and critically point dried cells as well as for live cells eventually.

X-ray lithography is a natural extension of contact micrography which was in vogue nearly two decades ago but which suffered for want of film resolution and greater X-ray source intensity. By contrast today, the method has great promise because of the development of grainless films (X-ray resists such as PMMA - polymethyl methacrylate - used here) and intense, potentially tunable X-ray sources as from a monochromater - synchrotron radiation source combination.[3]

In the present studies, as with those already reported, a 1 to μm thick film of PMMA has been spun onto a structureless support, a disk of highly polished silicon. This is our film and backing.

Two PMMA disks were coated with cells dried by the critical point method[4] using $CO_2$. One cell-resist open-faced sandwich was irradiated at the IBM Laboratories, Yorktown Heights, N.Y. with 4.48 nm Carbon $K_\alpha$ radiation for 25 hours with an exposure density $\sim 5 \times 10^3$ J/cm$^3$ behind the $\sim$1.5 μm thick cells. The second cell X-ray resist disk was irradiated with synchrotron radiation from the DESY, Hamburg electron synchrotron, reflected at a glancing angle of $4^\circ$ to eliminate wavelengths shorter than $\sim$ 2.5 nm. The effective exposure continuum lay between 2.5 and 4.4 nm while the radiation exposure density behind the cell is estimated to be $\sim 10^3$ J/cm$^3$. This and other experimental information is summarized in Table 1.

The amount of soft X-radiation reaching and damaging the resist in each case depends on both the density of atoms of a particular type, such as carbon, in the cell and their characteristic X-ray absorption spectra. Consequently, as we have changed (albeit crudely) the wavelength of the bombarding X-rays, one expects and does find a change in the replication pattern, giving information on the elemental composition of the heart of chick embryo cell under study.

Once irradiated, the cells were washed in ethyl alcohol and wiped with a Q-tip from the PMMA surface, in some cases leaving behind some cell fragments as seen in Fig. 1. The

irradiated resist was then developed at room temperature (Table 1). The rate of dissolution of the damaged and partly decomposed resist depends critically upon the amount of energy absorbed in the carbon and oxygen based PMMA resist. The relief replica in PMMA which remained after development was then coated with a 60:40 mixture of Au:Pd and read back with a commercial JEDL-35 SEM with a limiting resolution quoted to be 10 nm, a factor of two worse than the expected upper limit to resolution for X-ray replication.[2] In practice we have not reached the recommended resolution, in part because the resolution noticeably deteriorates as the metal coated PMMA replica is bombarded with 25-35 KeV electrons (note the lines in Fig. 1).

In Table II we summarize some of the results one obtains from the analysis of the micrographs in Fig. 1. It is clear from the table as well as from the figure that the resolution is worse for the replica made with 4.48 nm Carbon $K_\alpha$ radiation. It would appear that to a large extent this results from faster dissolution of the damaged PMMA resulting from the larger density of radiation absorbed by the PMMA in this case, thus leading to the loss in fine structure and the obvious over-development, for example, the microspikes along the edge of the cell and the blending together of structure along the main axis of the cell.

The two cell replicas shown in Fig. 1 were chosen for similarity in overall length ($\sim 80\,\mu$), length of nucleus ($\sim 10\mu$) and nucleolus ($\sim 2\mu$), and width of cell. Allowing for the

slight over-development of the replica made with 4.48 nm radiation, at 60$^{c}$ tilt of the SEM stage, one observes immediately the gross difference in the thickness of the replicas in the regions of the nucleus and nucleolus and the microtubules which run the length of the cell from the nuclear region out onto the lamellapodia and finally into the microspikes. This we suggest reflects a difference in chemical composition in these regions. It is significant that the nuclear structure is much more clearly defined for the replica made with the Carbon $k_{\alpha}$ radiation (4.6 - 4.8 nm). This probably reflects the preferential absorption in the L levels of phosphorus and perhaps sulphur, both of which have thresholds for 2s and 2p excitation just below the Carbon K onset. This is consistent with the fact that P concentrates in the region of the nucleus and the nucleolus, primarily in the RNA and DNA molecules. It is significant too, that the edge of the nucleolus is so pronounced, no doubt reflecting a build-up of the same elements in the membrane there. The prominent structure found[2] in the Carbon $K_{\alpha}$ replica of a chromosome from the salivary gland of Drosophila can also be explained by the preferential absorption of phosphorus in much the same way as we have observed it here.

In the near future soft X-ray lithographic techniques should facilitate both spectroscopic as well as microscopic studies of prepared cell mounts and cells in vitro. Our first attempts to replicate live cells have failed only because the cells have moved during the time it has taken to make the replica.

It is our pleasure to thank Profs. R.C. Buck and I.G. Walker for their continuing help in preparing cell samples and interpreting results. Particular thanks are given to Facultés Universitaires, Namur, Belgium who have assisted with the program.

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3. E. Spiller, D.E. Eastman, R. Feder, W.D. Grobman, W. Gudat, J. Tapalian, J. Appl. Phys. 47, 5450-5459, (1976)

4. Seven-day chick embryo heart fragments were grown for several days in Falcon plastic flasks in McCoy's 5A medium supplemented with 20% foetal calf serum containing antibiotics Cells were harvested by scraping them into a fresh medium with a rubber policeman. The coated silicon discs were overlaid with about 5 ml of cell suspension and incubated at $37^{o}$ C in a 5% $CO_2$ Air mixture. After 24 to 48 hours they were fixed in half strength Karnofsky fixative (Karnofsky, M.J.; J. Cell Biol. 27, 137A, [1965]) for one hour and then dehydrated in ethanol and dried by the critical point method using $CO_2$.

TABLE I EXPERIMENTAL CONDITIONS FOR TWO DIFFERENT IRRADIATIONS OF CELL - PMMA DISKS

| | IBM Carbon $K_\alpha$ 4.48 nm | DESY Synch.Rad. 4.5 2.5 nm | C/SR 150 |
|---|---|---|---|
| Radiation Time | 25 hr. | 10 min. | |
| Exposure Density | | | |
| Behind 1.5 μm cell J/cm$^3$ | $<5 \times 10^3$ | $\sim 10^3$ | <5 |
| Development Time | 1/2 min. | 1/2 min. | 1 |
| Developer Ratio IPA:MIBK | 3:1 | 1:1 | |
| Estimated Resolution | <50 nm | <20 nm | ~2.5 |
| Noise in Background points/μm$^2$ | 5-6 | 25-30 | 0.2 |

TABLE II COMPARISON OF RESULTS FOR TWO IRRADIATIONS (ALL DIMENSIONS IN μm)

(replicas in PMMA viewed at 60° tilt of SEM stage)

| | IBM Carbon K 4.48 nm | DESY SYNCH.RAD. 4.5-2.5 nm | C/SR |
|---|---|---|---|
| Maximum height of replica edge* | ∿0.5 | 0.5 | 1 |
| Maximum height of nucleus replica above cell replica * | ∿0.3 | <0.03 | >10 |
| Maximum height of nucleolus replica above nucleus replica * | ∿0.45 | <0.03 | >15 |
| Maximum height of ridges replica* | 0.1 - 0.15 | <0.03 | >3 |
| Maximum height of horizontal microspikes* | <0.07 | <0.28 | 0.25 |
| Maximum width of vertical microspikes | 0.03 - 0.07 | 0.12 - 0.15 | <0.5 |

* Corrected for Angle

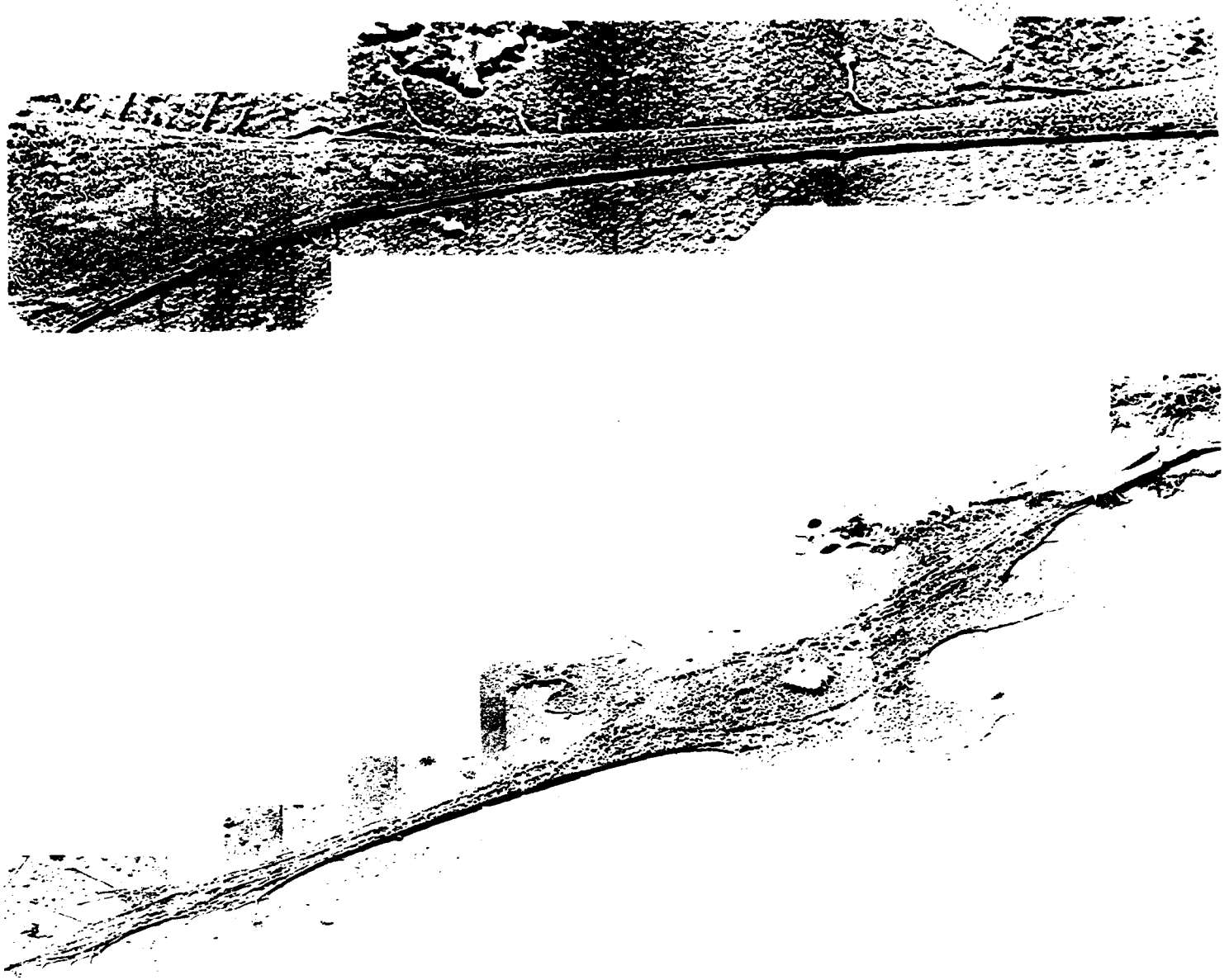

Fig. 1. Two micrographs of part of heart of chick embryo cells. In the upper left the cell was replicated using broad band (2.5 - 4.5 nm) synchrotron radiation while in the lower part of the figure the replication was made with 4.48 nm Carbon $K_\alpha$ radiation. The prominence of the nucleus and nucleolus in the Carbon $K_\alpha$ replica reflects the build-up of phosphorous in this region of the cell.

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